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Nicotinic Regulation of c-*fos* and Osteopontin Expression in Human-Derived Osteoblast-Like Cells and Human Trabecular Bone Organ Culture

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Long-term in vivo studies have highlighted smoking as a risk factor in postmenopausal osteoporosis, bone fracture incidence, and increased nonunion rates. In contrast, there are few data postulating the effects of smoking at the cellular level in human skeletal tissue. In this study, we present novel evidence demonstrating that the nicotinic receptor α4 subunit is present in human primary bone cells by using reverse transcriptase-polymerase chain reaction (RT-PCR). In addition, we demonstrate direct cellular effects of nicotine on primary human bone cells and blockage of these effects with a nicotinic receptor antagonist, D-tubocurarine. Nicotine effects on cell proliferation were biphasic with toxic, antiproliferative effects at high levels of nicotine (>1 mmol/L) and stimulatory effects at very low levels (0.01–10 μmol/L) after 72 h. This nicotine-induced increase in cell proliferation was inhibited in a dose-dependent manner by the addition of D-tubocurarine. In addition, proliferation effects from low-level treatment correlated with an upregulation of expression of the AP-1 transcription factor, c-*fos*, within 1 h, which was blocked by incubation with D-tubocurarine. To determine in situ bone cell responses within their trabecular matrix, cores of human bone isolated from biopsies were perfused with 0.1 μmol/L nicotine for 24 h. Western analysis of proteins isolated from the cores highlighted an increase in osteopontin, a bone matrix protein implicated in regulating resorption, which was partially inhibited by the addition of D-tubocurarine. To conclude, our results suggest that nicotine has a direct effect on human bone cells in modulating proliferation, upregulation of the c-*fos* transcription factor, and the synthesis of the bone matrix protein, osteopontin. (Bone 28: 603–608; 2001)

Key Words: Nicotine; Osteoblasts; Human; Nicotinic receptor; c-*fos*; Osteopontin.

Introduction

In vivo studies have highlighted an important role for tobacco and tobacco smoke in many skeletal diseases. Smoking has been implicated as a risk factor in postmenopausal osteoporosis with effects on bone content and risk of fracture.[1,8,32] Smokers have been shown to have 4%–5% lower bone mineral density (BMD) values, which was shown to be significant in the lumbar spine.[25] An investigation into the rate of bone change demonstrated a greater loss in smokers compared with nonsmokers.[18,19] Furthermore, several studies of tibial fractures have resulted in unexpectedly long healing times and increases in nonunion rate apparently associated with smoking.[20] Although there have been many in vivo studies highlighting the effect of smoking on human skeletal tissue, there have been relatively few in vitro studies attempting to understand the action of nicotine at the cellular level.

Nicotine is a major component of the particulate phase of all cigarette smoke and, controversially, is the drug that causes addiction. Nicotine is known to stimulate the central nervous system by increasing heart rate and blood pressure. Nicotinic acetylcholine receptors found in neuronal, muscle, epithelium, tendon, and sensory cells from a number of species, including humans, are transmitter-gated ion channels and are formed from five homologous subunits surrounding a central cation channel.[4,11,22,29,31,33,35] Although calcium, sodium, potassium, and chloride channels have been identified in primary bone cells and bone cell lines, no studies have yet been published characterizing the nicotinic receptor in human bone cells.

In this study, we describe the presence of the nicotinic acetylcholine receptor subunit mRNA using polymerase chain reaction. We provide evidence for direct and receptor-mediated short-term effects of nicotine on primary human cell proliferation and AP-1 transcription factor, c-*fos*, gene expression. Finally, we describe in situ studies using a human bone core perfusion model that support our observations of receptor-mediated nicotinic effects on human skeletal tissue.

Methods

Human Bone Cell Culture

Human osteoblast cells were cultured essentially following the procedure described by Kassem et al.[17] Briefly, small fragments of bone were isolated from trabecular bone biopsies taken from patients being treated at Accident and Emergency, North Staffordshire Hospital, as described in what follows. Patients were predominantly nonsmokers with tissues derived from smokers used for preliminary comparisons. Bone fragments were then transferred to flasks containing α-minimal essential medium

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(α-MEM), 10% fetal calf serum (FCS), 1% L-glutamine, and 1% antibiotics, and cultures of proliferating osteoblasts were observed after 21 days (37°C, 5% CO_2 incubator). Osteoblastic phenotype was determined in cell populations by staining for alkaline phosphatase enzyme marker. In addition, phenotype of cultures was determined using reverse transcriptase-polymerase chain reaction (RT-PCR) for collagen type I, and cbfa1, bone cell differentiation factor mRNA expression (data not shown). In some experiments, the mouse osteoblastic cell line, MG63, was used. Culture media and feeding protocols were as described earlier for primary bone cell cultures.

RT-PCR Analysis for α4 Nicotinic Receptor Subunit

RNA was isolated from primary human osteoblasts, the MG63 osteoblastic-like cell line, human bone cores, and human brain tissue using a modified Chomczynski method.[6] cDNA was obtained by RT and subjected to PCR amplification using primers designed for the α4 neuronal nicotinic receptor mRNA sequence (forward primer TGTCCATCGCTCAGCTCATT, reverse primer CATGTTCACCAGGTCGATCT, product size 380 base-pairs). PCR reactions were carried out in 25 μL volumes with: 200 μmol/L each of dATP, dGTP, dTTP, and dCTP; 2 pmol of each primer template DNA; and 1 U of Taq-DNA polymerase. PCR amplification occurred at an annealing temperature of 56°C after 40 cycles. The PCR products (5 μL) were visualized on an agarose gel (containing ethidium bromide) with ultraviolet light.

Cell Proliferation Studies

Cells seeded at 10^4 cells/cm^2 were used to test the effects of nicotine (range 0.1 pmol/L to 10 mmol/L; free base; Sigma, UK) and the nicotinic receptor antagonist D-tubocurarine on human primary osteoblast proliferation. Initially, a dose response to the inhibitor was carried out between the range of 10 nmol/L and 10 mmol/L. The optimal dose used for further study was of an equivalent concentration to that used by other researchers in studies of the pharmacology of neuronal nicotinic receptor subtypes.[12] After cell adherence, various nicotine and D-tubocurarine concentrations were added to the media with 2% FCS and culture conditions were maintained for 72 h (37°C, 5% CO_2 incubator). [Methyl-^{3}H]-thymidine (1 μCi/mol, specific activity 10 Ci/mmol; ICN) was also added to the medium for incorporation by proliferating cells. After 72 h, the cells were trypsinized and harvested onto Titertek cell harvester filter mats (ICN). [Methyl-^{3}H]-thymidine uptake in cells was assessed using a Beckman LS-1801 scintillation counter.

*RT-PCR Analysis of c-*fos

Human primary osteoblast cells were plated at a concentration of 10^{-4} cells/cm^2 and cultured until confluent. Twenty-four hours prior to nicotine treatment, the medium was replaced with 2% fetal calf serum (FCS). Following treatment for 1 h (see later), the medium was removed and the cells resuspended the primary solution for RNA extraction. cDNA was prepared by RT and subjected to PCR with c-*fos* primers (forward TCTCTTACTACCACTCACCC, reverse TGGAGTGTATCAGTCAGCTC). Control reactions were prepared for the HPRT housekeeping gene using primers (forward TTGTAGCCCTCTGTGTGCTCAAG, reverse GCCTGACCAAGGAAAGCAAAGTC).

Both PCR reactions were carried out in 25 μL volumes with 200 μmol/L each of dATP, dGTP, dTTP, and dCTP; 2 pmol of each primer template DNA; and 1 U of Taq DNA polymerase. PCR amplification occurred with an annealing temperature of 57°C after 40 cycles. The PCR products (5 μL) were visualized on an agarose gel (containing ethidium bromide) with ultraviolet light.

Initially, a time course experiment was conducted. c-*fos* expression was monitored at 30 min, 1 h, and 1.5 h following treatment with 0.1 μmol/L nicotine, and found to be maximal at 1 h (data not shown). This timepoint was then used throughout the experimentation. In addition, a dose response was carried out using a control with vehicle only, and with 0.01, 0.1 and 1.0 μmol/L nicotine added to the medium. All three doses resulted in c-*fos* expression at 1 h. Subsequently, 0.1 μmol/L was used for further study to determine whether the response could be blocked by the nicotinic inhibitor, 1 μmol/L D-tubocurarine. Osteoblasts were treated with 0.1 μmol/L nicotine, 0.1 μmol/L nicotine with 1 μmol/L D-tubocurarine, or vehicle-only controls.

Human Trabecular Bone Core Perfusion Model

Human bone cores were isolated with a trephine from routine pinhole sites required for the addition of an external fixator to a tibial fracture from trauma patients (aged 25–60) being treated at the Accident and Emergency, North Staffordshire Hospital. The cores were measured, weighed, and divided into two or three equal parts not exceeding 5 mm in length (one section as a internal control, the other sections for treatment). The cores were placed in a perfusion chamber consisting of a pump, a media reservoir, and an incubation chamber, all linked with autoclavable tubing to a Watson multichannel peristaltic pump. This procedure was previously described in detail for canine bone cores by El Haj et al.[19] and adapted from their protocol. Cores were treated with either low FCS medium, medium containing 0.1 μmol/L nicotine, medium containing 0.1 μmol/L nicotine and 1 μmol/L D-tubocurarine, or vehicle-only controls. The cores were perfused at a rate of 1 mL/min for 24 h to assess the production of the bone matrix protein, osteopontin, by western analysis. In addition, cores were perfused for 1 h to analyze the effect of nicotine on c-*fos* expression (RNA isolation and RT-PCR analysis of c-*fos* described in earlier methods).

Protein Analysis

For sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), cells in bone cores were lysed by the addition of 100 μL phosphate-buffered saline (PBS) containing 0.1% Triton X-100. Samples of lysate were mixed with equal volumes of dissociation buffer (0.18 mol/L Tris-HCl [pH 6.8], 3% w/v SDS, 30% v/v glycerol, 15% v/v β-mercaptoethanol, and 0.01% w/v bromophenol blue), boiled for 4 min, and subjected to electrophoresis in 12.5% acrylamide gels in an electrophoresis (Bio-Rad Mini Protean II) apparatus. Proteins were either transferred to Hybond-PVDF membrane using a Mini Transblot (Bio-Rad) cell overnight at 30 V or stained with Coomassie blue to determine equal loading. Transfer to Hybond-PVDF was confirmed by staining gels with Coomassie blue.

Enhanced chemiluminescence (Pierce, UK) was used to detect proteins of interest. Antisera against rat osteopontin (mouse monoclonal; Developmental Studies Hybridoma Bank, Johns Hopkins University, Baltimore, MD) and vinculin (mouse monoclonal; Sigma) were used at a dilution in PBS of 1:2000. Secondary anti-mouse monoclonal antibodies conjugated with peroxidase were diluted to 1:4000. Bands were scanned and intensities evaluated using a Black Widow 4800 PP scanner. Western blots were stripped and reprobed. Briefly, the membrane was submerged in stripping buffer (100 mmol/L β-mercaptoethanol, 2% [w/v] SDS, 62.5 mmol/L Tris-HCl [pH 6.7]) and incubated at 60°C for 30 min with occasional agitation. The mem-

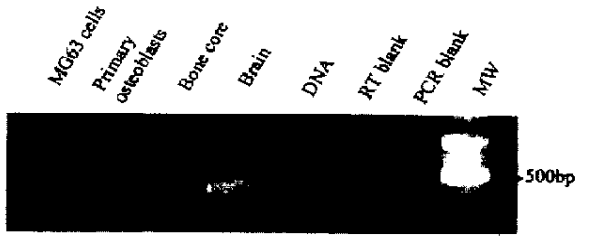

Figure 1. RT-PCR analysis for the presence of α4 acetylcholine nicotinic receptor subunit in a variety of human skeletal tissue and cell types. α4 acetylcholine nicotinic receptor subunit was observed in human primary osteoblasts, human osteoblast-like cell line MG63, isolated human bone cores, and in a control tissue (human brain). Primers were designed as described in *Methods*.

brane was then washed for 2 × 10 min in PBS-Tween at room temperature.

Statistical Analysis

All values are expressed as mean ± standard deviation unless stated otherwise. Quantitative analysis was carried out using Student's *t*-test and analysis of variance.

Results

α4 Nicotinic Receptor in Bone Tissue

Using primers designated to the α4 subunit, RT-PCR amplified the acetylcholine nicotinic receptor subunit in human primary osteoblasts, MG63 osteoblastic cell line, human bone cores, and human brain (**Figure 1**). PCR products of 380 bp were sequenced and revealed complete homology with the α4 nicotinic receptor from brain in all of the amplified DNA bands (data not shown). No difference was observed in the levels of α4 nicotinic receptor mRNA between bone cells derived from smokers and from nonsmokers using RT-PCR.

Effect of Nicotine and D-Tubocurarine (Nicotinic Receptor Inhibitor) on Human-Derived Osteoblasts in Culture

Nicotine treatment (1 pmol/L to 1 mmol/L) has a dose-dependent effect on proliferation in primary human osteoblasts in culture (**Figure 2**). Addition of nicotine at concentrations of 0.1 to 1

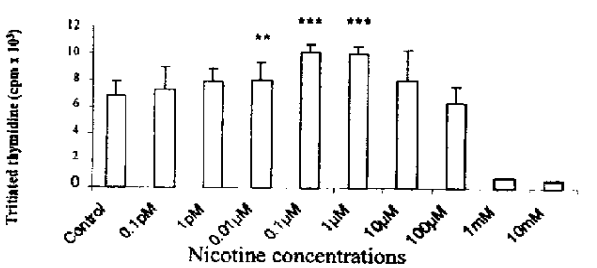

Figure 2. The effect of a variety of doses of nicotine (ranging from 0.1 pmol/L to 10 mmol/L) on proliferation of human primary osteoblasts. Incorporation of tritiated thymidine was measured 48 h after the onset of nicotine incubation. Values are mean ± SD of six replicates from three patients. Statistical values calculated by analysis of variance: $^{**}p < 0.01$ and $^{***}p < 0.001$.

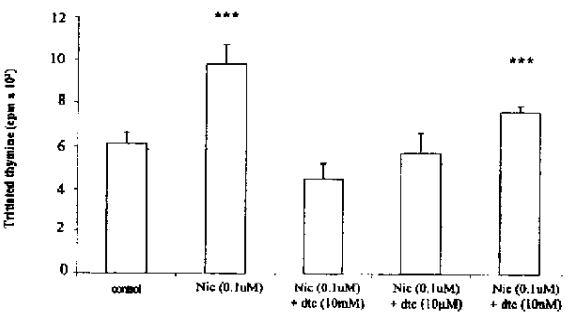

Figure 3. The effect of 10 nmol/L, 10 μmol/L, and 10 mmol/L D-tubocurarine (nicotinic receptor antagonist) on proliferation of 0.1 μmol/L nicotine-stimulated human primary osteoblasts. Incorporation of tritiated thymidine was quantified 48 h after the onset of nicotine/D-tubocurarine incubation. Values are mean ± SE of six replicates from three treatments. Statistical values calculated by Student's paired *t*-test. $^{***}p < 0.001$.

μmol/L significantly increased osteoblast proliferation rate above vehicle-only controls (tritiated thymidine counts, vehicle control 68,291.1 ± 1050; 0.1 μmol/L nicotine 10,088.8 ± 624, n = 3, $p < 0.001$; 1 μmol/L nicotine 9973 ± 580, n = 3, $p < 0.001$). Incubation of cells with nicotine concentrations of >1 μmol/L resulted in a decrease in cell proliferation and, ultimately, cell death. Similar trends were observed at the timepoints of 24, 48, and 72 h and no changes in the trend were observed between smokers and nonsmokers (data not shown).

The addition of D-tubocurarine, a nicotinic receptor inhibitor, to nicotine-stimulated cells (0.1 μmol/L) resulted in a dose-dependent decrease in human osteoblast proliferation (**Figure 3**). Also, 0.1 μmol/L nicotine (tritiated thymidine counts 9806 ± 921) and 0.1 μmol/L nicotine with 10 nmol/L D-tubocurarine (7626 ± 318 cpm) significantly increased cell proliferation in comparison to vehicle-only control cells (6107 ± 523 cpm). Inhibition of 0.1 μmol/L nicotine-induced cell proliferation was observed upon incubation of cells with 10 μmol/L and 10 mmol/L D-tubocurarine.

Effect of Nicotine and D-Tubocurarine on c-fos Expression

Incubation of confluent human primary osteoblast cells with 0.1 μmol/L nicotine resulted in upregulation of c-*fos* after 1 h (n = 5) (**Figure 4a**). The 0.1 μmol/L nicotine-induced c-*fos* expression was inhibited in the presence of 1 μmol/L D-tubocurarine after 1 h (Figure 4b). Control cells treated with vehicle only did not upregulate c-*fos* expression (n = 5) (Figure 4a), demonstrating that the response was not due to mechanical perturbation by the addition of agents. Differences in responses between cells derived from smokers and nonsmokers were not observed (data not shown).

Perfusion of Human Bone Cores With Nicotine and D-Tubocurarine

Increased levels of the bone matrix protein, osteopontin, were observed in human bone cores perfused with 0.1 μmol/L nicotine for 24 h (n = 3) (**Figure 5a,b**). The nicotine-induced increase in osteopontin was partially inhibited by 1 μmol/L D-tubocurarine, after 24 h perfusion (Figure 5a). Perfusion of cores for 1 h with nicotine resulted in upregulation of c-*fos* expression as described for primary cultures and 1 h treatment with nicotine in the

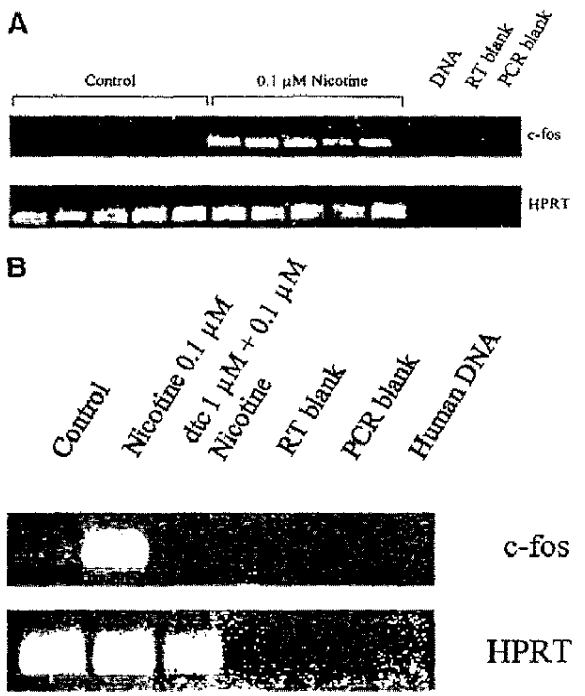

Figure 4. (a) RT-PCR analysis of c-*fos* mRNA levels in control and 0.1 μmol/L nicotine-stimulated confluent human primary osteoblasts (n = 5). RNA extraction was carried out 1 h after nicotine incubation. RT-PCR of the housekeeping gene, HPRT, was included to ensure measurable quantities of cDNA in all samples. HPRT and c-*fos* PCR products are visualized by ethidium bromide staining of 1% agarose gels. (The more sensitive silver-stained polyacrylamide gel method of visualizing PCR products confirms no c-*fos* expression in control samples [data not shown]). (b) The effect of 0.1 μmol/L nicotine and 0.1 μmol/L nicotine with 1 μmol/L D-tubocurarine on c-*fos* mRNA expression in confluent human primary osteoblasts. RT-PCR analysis demonstrates levels of housekeeping gene HPRT in all test samples. c-*fos* expression observed in nicotine-stimulated cells only.

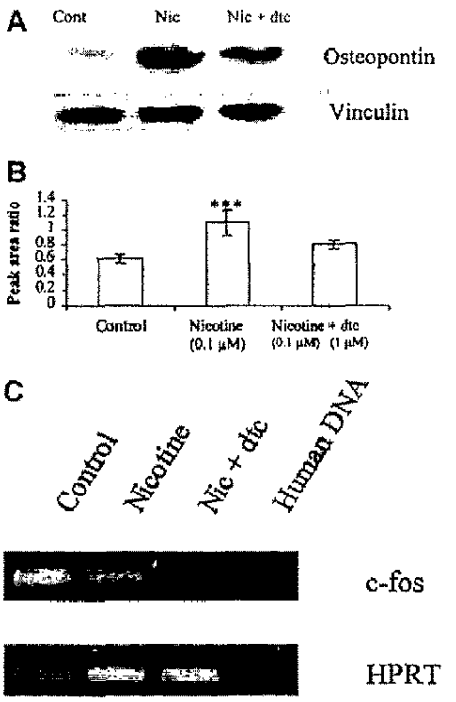

Figure 5. (a) Western analysis of proteins extracted from trephine-isolated trabecular bone cores. Each bone core was divided into two equal parts, one part perfused for 24 h with media and low FCS (see *Methods*) and one part perfused with media containing 0.1 μmol/L nicotine or 0.1 μmol/L nicotine/1 μmol/L D-tubocurarine. (a) Osteopontin binding to nitrocellulose blot of nicotine-treated cores and controls is shown in Figure 4a. The membrane was stripped and reprobed with an antiserum for vinculin. (b) Following scanning densitometry, the peak area ratios of osteopontin to vinculin bands are plotted against each treatment (n = 12, ***p = 0.005 by ANOVA). (c) Trabecular cores treated with 0.1 μmol/L nicotine or with 0.1 μmol/L nicotine/1 μmol/L D-tubocurarine or control with vehicle. After 1 h, RNA was isolated from the cores and c-*fos* expression analyzed using RT-PCR. RT-PCR of the housekeeping gene, HPRT, was described to ensure measurable quantities of cDNA in all samples. HPRT and c-*fos* PCR products are visualized by ethidium bromide staining of 1% agarose gels.

presence of D-tubocurarine resulted in an inhibition of c-*fos* expression (Figure 5c).

Discussion

The present study is the first to demonstrate, in human bone tissue, the presence of the α4 neuronal nicotinic acetylcholine receptor (nAChR)-subunit transcript characteristic of nicotinic receptors, and has further demonstrated nicotine-dependent effects of c-*fos* induction and osteopontin upregulation.

The effect of smoking and key byproducts, such as nicotine, have been shown to have a major effect on skeletal remodeling; however, only one recent report[30] has described the presence of nicotinic acetylcholine receptor subunits in fetal chick connective tissues during development. Romano et al.,[30] using immunohistochemical techniques, demonstrated expression of the nicotinic receptor α7 mRNA in differentiating periosteal tissue from chick embryos at stage 11. However, these investigators suggested that fibroblastic cells within embryonic tissue might have been responsible for the expression of the α7 receptor.

nAChRs are widespread throughout the central and peripheral nervous system and function as ligand-gated ion channels that rapidly desensitize in response to nicotine insult.[37] The α4 nAChR subunit has been localized in a variety of neuronal and nonneuronal tissues, including human brain,[25] human retina,[15] human nasal epithelium,[4] chick sympathetic ganglia,[23] rat dorsal ganglia,[38] and developing chick muscle.[7] In this study, we have demonstrated expression of the α4 neuronal nicotinic acetylcholine transcript in the human primary osteoblast, human trabecular bone biopsies from tibial fracture repair patients, and the osteoblast-like MG63 cell line as assessed by RT-PCR.

Studies involving the direct effects of nicotine, the major metabolite of cigarette smoke, have mainly investigated the effects on immortalized bone cell lines in vitro or nonhuman species. In the present study, we have described the effects of nicotine on primary human isolated osteoblasts and perfused human bone cores where cells are localized within their natural matrix using a procedure similar to that described by El Haj et al.[9] using canine bone cores. The levels of nicotine used in this study incorporated the concentrations of nicotine observed in circulation of habitual cigarette smokers (0.06–0.3 μmol/L)[3] and saliva levels of long-term snuff users (0.6–9.6 μmol/L).[15]

In the isolated primary cell culture, there were bimodal effects on cell proliferation. Low levels of nicotine (0.1–1 μmol/L) increased cell proliferation at 24, 48, and 72 h. Exposure to high levels of nicotine (< 1 mmol/L) resulted in a dramatic decrease in cell proliferation and, ultimately, cell death. These observations are supported in part by Fang et al.[10] who studied the effect of high levels of nicotine on proliferation of osteoblast-like cells and showed that incubation of cells with levels of nicotine >1 μmol/L resulted in a decrease in proliferation. Interestingly, incubation with 1 μmol/L nicotine reduced cell proliferation by 40% compared with controls, differing from our observations in primary human osteoblast cells. This apparent difference in cellular proliferation may reflect the differing response to nicotine in cell lines and primary human tissue.

Lenz et al.[21] demonstrated an increase in tritiated thymidine incorporation when osteoblast-like cells from embryonic chick calvariae were induced with nicotine. Similarly, nicotine has been shown to stimulate DNA synthesis and proliferation in vascular endothelial cells[34] in a bimodal manner similar to that described in our study. Furthermore, nicotine has been shown to inhibit collagen and alkaline phosphatase activity, but stimulate DNA synthesis (suggesting increased cell turnover) in an osteoblast cell line.[27] However, response to nicotine stimulation described in all three studies was observed after cells were induced with nicotine for <72 h. Long-term effects over weeks may reverse these enhanced proliferative effects.

Short-term effects of nicotine on expression of the c-*fos* oncogene, a member of the AP-1 family of transcription factors, was also measured. c-*fos* expression is rapidly induced by mitogenic signals such as growth sera and growth factors as well as mechanical and stress responses.[2] Incubation of human-derived primary osteoblasts resulted in an increase in c-*fos* expression in subconfluent and confluent cells, suggesting nicotine-stimulated cellular proliferation. Using RT-PCR, expression of the c-*fos* transcript in confluent noninduced cells was undetectable; however, stimulation of cells with nicotine (0.1 μmol/L) resulted in an increase in c-*fos* transcript expression.

The nicotine-induced increase in cellular proliferation can be inhibited in a dose-dependent manner by incubation of primary cells with D-tubocurarine, a nicotinic acetylcholine receptor antagonist. D-tubocurarine is an alkaloid that binds to all nicotinic receptor subtypes.[5,12] The nicotine-induced elevation in c-*fos* expression was blocked by D-tubocurarine, suggesting the involvement of nicotinic acetylcholine receptors in the nicotine-c-*fos* response. This provides further evidence for nicotine acting in situ at the cellular level through nicotinic receptors.

In addition to these studies on isolated cells in culture, we have investigated the effect of nicotine, and its blocker, D-tubocurarine, on perfused human trabecular bone biopsies. In this system, cells remain in situ in their surrounding matrix. Increased osteopontin protein levels were observed in human bone cores perfused with nicotine, whereas a reduction in osteopontin protein expression was observed in the presence of D-tubocurarine. Osteopontin has been demonstrated to be an indicator of bone turnover and also degradation.[28] The elevated expression of this protein would correlate with nicotine effecting bone turnover and, after long-term administration, possibly result in loss of skeletal tissue or decline in the rate of bone production during fracture repair.

Nicotine (which is eventually broken down into cotinine in vivo) is maintained at a high level by habitual smoking, and has been shown to have inhibitory proliferation effects in vivo on bone turnover.[8,18] Habitual smokers often suffer loss of bone mass,[16,18] increased risk of fracture,[18,19] prolonged fracture repair, and increased nonunion rates.[20] The levels of nicotine in bone have not yet been measured accurately and may be accumulated into the matrix and released during degradation. It is possible that circulating levels around the fracture site may exceed those measured in the blood from smokers. Our results did not identify differences in the absence or presence of receptor subunit expression from bone cells derived from smokers and nonsmokers. Also, we did not detect differences in responses to nicotine from cells derived from smokers and nonsmokers. This could indicate that levels of receptors may not vary with habitual smokers vs. nonsmokers; however, further quantitative studies of the levels of receptor expression are needed to confirm these findings.

In summary, we have demonstrated the presence of the nicotinic receptor subunit α4 in primary human bone cell cultures and human bone biopsies. Nicotine increases the proliferative rate and c-*fos* transcript expression in human primary osteoblasts. These observations have been extended to in situ studies in perfused human trabecular cores where nicotine induces c-*fos* transcript expression and osteopontin expression. Further support for a receptor-mediated process is demonstrated by our finding that the nicotinic receptor antagonist, D-tubocurarine, inhibits nicotine-induced responses.

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